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Dual apoptotic DNA fragmentation system in the fly: Drep2 is a novel nuclease of which activity is inhibited by Drep3

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ABSTRACT

DNA fragmentation is the hallmark of apoptotic cells and mainly mediated by the DNA fragmentation factor DFF40(CAD)/DFF45(ICAD). DFF40 is a novel nuclease, whereas DFF45 is an inhibitor that can suppress the nuclease activity. Apoptotic DNA fragmentation in the fly is controlled by four DFF-related proteins, known as Drep1, 2, 3 and 4. However, the functions of Drep2 and Drep3 are totally unknown. Here, we found that Drep2 is a novel nuclease whose activity is inhibited by Drep3 through a tight interaction with the CIDE domain. Our results suggest that the fly has dual apoptotic DNA fragmentation systems: Drep1:Drep4 and Drep2:Drep3 complexes.

Structured summary of protein interactions:
Drep2 CIDE and Drep-3 CIDE bind by blue native page (View interaction)
Drep2 CIDE and Drep-3 CIDE bind by molecular sieving (View interaction)

1. Introduction

Apoptosis is an orderly cellular suicide process in response to various stimuli [1,2] and failure to control apoptosis can cause serious human diseases such as cancer, autoimmune disorders, and Alzheimer's disease [3–6]. Characteristic changes in chromosomes, such as chromatin condensation and cleavage, are hallmarks of apoptosis and are often used as a tool to detect apoptotic cell death [7–9]. Previous studies have revealed that apoptotic DNA fragmentation is mediated by a novel DNA fragmentation factor (DFF), which is composed of a heterodimeric complex called DFF40 (CAD: mouse homologue):DFF45 (ICAD: mouse homologue). The DFF40:DFF45 complex was first identified as dependent on caspase-3 activity in the human Hela S-100 fraction [8]. DFF40 is a novel nuclease with a nuclear localization signal, whereas DFF45 is an inhibitor that suppresses DFF40 nuclease activity via tight interaction. Interestingly, DFF45 seems to function as a chaperone for DFF40 during its synthesis [7,10,11]. When effector caspases such as caspase-3 are activated by apoptotic stimuli, they cleave DFF45, allowing DFF40 to enter the nucleus and degrade chromosomal DNA [9,12]. During this process, DNA undergoes fragmentation and is eventually cleaved into regular ~180 bp fragments [8,9].

Apoptosis and apoptotic DNA fragmentation is conserved among different species including *Drosophila melanogaster*, and several homologous proteins of the DFF40:DFF45 complex have been identified [13]. Unlike the mammalian system, apoptotic DNA fragmentation in the fly is controlled by four DFF-related proteins known as Drep1 (dDFF45), Drep2, Drep3, and Drep4 (dDFF40) [13]. Drep1 and Drep4 are DFF45 and DFF40 homologues, respectively [14]. Although no clear evidence has been presented, Drep2 and Drep3 are regulators of the DFF system during apoptosis in the fly [13]. All four proteins have a conserved CIDE domain, which is involved in protein–protein interactions [15]. The CIDE domain-containing proteins, called CIDE family proteins, have been detected in many mammalian systems as well as the fly [16], but the function of these proteins, including Drep2 and Drep3 are not clear.

To elucidate the possible function of Drep2 and Drep3 and understand the mechanism of apoptotic DNA fragmentation in the fly system, we purified Drep2, Drep3, and their CIDE domains and performed a biochemical study. As a result, we found that full-length Drep2 exists as a highly oligomeric form mediated by CIDE domain oligomerization in solution unlike Drep3, which usually exists as a monomer in solution. We also found that Drep2 has nuclease activity, and that Drep2 nuclease activity was inhibited by Drep3 by a direct interaction. Based on our results, we conclude that the fly has a dual apoptotic DNA fragmentation system: a Drep1:Drep4 complex and a Drep2:Drep3 complex. Drep4 and

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Drep2 are nucleases and their activity is inhibited by Drep1 and Drep3, respectively.

2. Materials and methods

2.1. Protein expression and purification

Full-length Drep1 (amino acid residues: 1–297), Drep2 (amino acid residues: 1–481) and Drep3 (amino acid residues: 1–242) were amplified by polymerase chain reaction (PCR) using gene-specific primers containing the NdeI and XhoI sites. The PCR fragments were subsequently digested and ligated into the pOKD5 home-made vector for Drep1 and Drep3 and the pET26b vector for Drep2. PCR fragments for the CIDE domains of Drep1 corresponding to amino acids 1–90, Drep2 corresponding to amino acids 1–84, and Drep3 corresponding to amino acids 112–195 were similarly prepared and cloned into the pOKD vector for Drep3 CIDE, the pER28a vector for Drep1 CIDE, and the pET26b vector for Drep2 CIDE.

All recombinant proteins were expressed in *Escherichia coli* BL21 (DE3) and purified by quick two-step chromatography. Expression was induced by treating the bacteria with 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) overnight at 293 K. The bacteria were then collected, resuspended, and lysed by sonication in 50 ml lysis buffer (20 mM Tris–HCl pH 7.9, 500 mM NaCl, and 10 mM imidazole). The bacterial lysate was then centrifuged at 16,000 rpm for 1 h at 4 °C. The supernatant fraction was applied to a gravity-flow column (Bio–Rad, Hercules, CA, USA) packed with Ni-NTA affinity resin (Qiagen, Valencia, CA, USA). The unbound bacterial proteins were removed from the column with a washing buffer (20 m*M* Tris–HCl pH 7.9, 500 m*M* NaCl, 60 mM imidazole, and 10% glycerol). The target proteins were eluted from the column using an elution buffer (20 m*M* Tris–HCl pH 7.9, 500 mM NaCl, and 250 mM imidazole). Protein purity was further improved using a Superdex 200 gel filtration column (Pharmacia, Piscataway, NJ, USA) pre-equilibrated with a buffer containing 20 mM Tris–HCl pH 8.0 and 150 mM NaCl.

2.2. Complex assay using gel-filtration chromatography

Purified Drep2 CIDE was mixed with a molar excess of Drep3 CIDE and applied to a gel-filtration column (Superdex 200 HR 10/30, GE Healthcare), which was pre-equilibrated with 20 mM Tris–HCl, pH 8.0, and 150 mM NaCl. The fractions were collected and subjected to SDS–PAGE.

2.3. Native PAGE shift assay

The protein interaction between Drep2 CIDE and Drep3 CIDE was monitored by native (non-denaturing) PAGE on a PhastSystem (GE Healthcare) with pre-made 8–25% acrylamide gradient gels (GE Healthcare). Separately purified proteins were pre-incubated at room temperature for 1 h before loading the gel. Coomassie Brilliant Blue was used for staining and detecting shifted bands.

2.4. Pull-down assay

A co-expression system was used to perform the pull-down assay. Drep2 CIDE in pET26b and Drep2 CIDE with no-tag in the

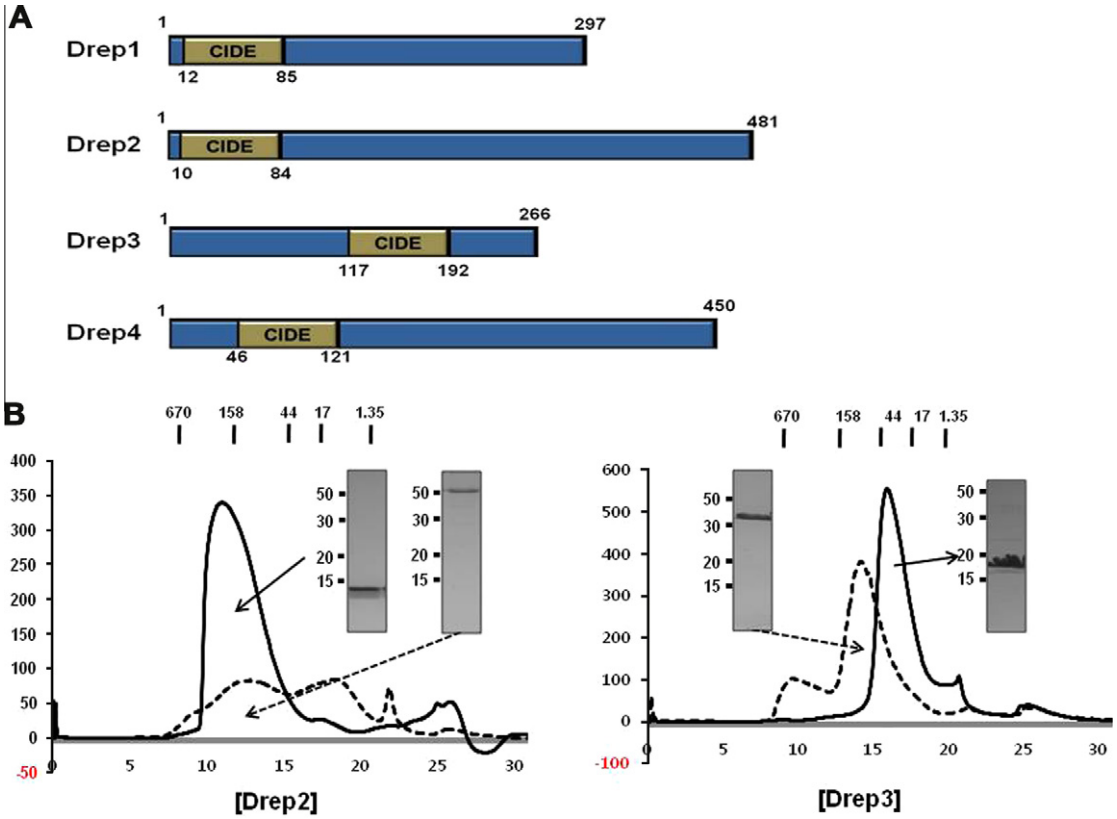

Fig. 1. Purification of full-length Drep2 and Drep3 and their CIDE domains. (A) Domain boundaries of Drep1, 2, and 3. Each CIDE domain is colored in gray and the residue number of amino acid corresponding to the CIDE domain is indicated. (B) Gel-filtration chromatograms and fractions of full-length Drep2 and its CIDE domain. Profile obtained from the full-length Drep2 in 150 mM NaCl and Tris buffer pH 8.0 is shown as a black dot. Profile obtained from the CIDE domain of Drep2 in 150 mM NaCl and Tris buffer pH 8.0 is shown as a black line. SDS–PAGE stained by Coomassie Blue from the peak gel-filtration chromatography fraction is shown at the upper right side. (C) Gel-filtration chromatograms and fractions of full-length Drep3 and its CIDE domain. Profile obtained from the full-length Drep2 in 150 mM NaCl and Tris buffer pH 8.0 is shown as a black dot. Profile obtained from the Drep3 CIDE domain in 150 mM NaCl and Tris buffer pH 8.0 is shown as a black line. SDS–PAGE stained by Coomassie Blue from the peak fraction of gel-filtration chromatography is shown next to each peak.

pOKD vector were co-transformed separately into BL21(DE3) *E. coli* competent cells. The expression was induced with 0.5 mM IPTG overnight at 20 °C. The cells were collected and lysed by sonication in lysis buffer (20 mM Tris buffer at pH 7.9, 500 mM NaCl, and 10 mM imidazole). The lysate was then removed by centrifugation, and the supernatant fractions were applied to gravity-flow column (Bio-rad) packed with Ni-NTA affinity beads (Qiagen). The unbound bacterial proteins were removed from the column with a washing buffer (20 mM Tris–HCl at pH 7.9, 500 mM NaCl, 60 mM imidazole, and 10% glycerol). The stable complex was detected by SDS–PAGE.

2.5. Nuclease activity assay

Purified Drep1, 2, and 3 (10 μg) were preincubated with 4 μg of linearized plasmid DNA at 37 °C for 1 h in a final volume of 20 μl in a buffer containing 20 mM Hepes pH 7.4, 5 mM $MgCl_2$ 50 mM NaCl, and 5 mM EGTA. The reaction was run on a 2% agarose gel for 30 min at 150 V. Purified Drep1 or Drep3 was mixed with Drep2 at 20 °C for 30 min before adding plasmid DNA for the inhibition assay.

3. Results and discussion

3.1. Purification and characterization of full-length Drep2 and Drep3 and their CIDE domains

DNA fragmentation is the hallmark of apoptotic cell death and is mainly performed by the DNA fragmentation factor DFF40:DFF45 complex. Four DFF-like proteins occur in the fly: Drep1, Drep2, Drep3, and Drep4. All three proteins, which were used in our study, have a well conserved CIDE domain that is involved in protein–protein interactions (Fig. 1A). Although Drep1 and Drep4 are DFF45 and DFF40 homologues, respectively, the function of Drep2 and Drep3 is still unclear. As a first step to elucidate the function of Drep2 and Drep3, we expressed and purified each CIDE domain of Drep 2 and Drep3 as well as the full-length proteins. Drep4 was not expressed in *E. coli*, whereas Drep1, Drep2, Drep3, and each CIDE domain were expressed well in *E. coli* as C-terminal His-tag fusion proteins and purified by Ni affinity chromatography followed by gel-filtration chromatography at 4 °C (Fig. 1B and C). Interestingly, full-length Drep2 and Drep2 CIDE seemed to be in a highly oligomeric state in solution unlike Drep3 CIDE, judging by the gel-filtration chromatography (Fig. 1B and C). Drep2 and Drep2 CIDE eluted in 10 ml on a Superdex 200 gel-filtration column, which corresponded to a size of >150 kDa (Fig. 1B). Drep3 and Drep3 CIDE eluted at approximately 15 and 16 ml respectively (Fig. 1C). Based on this preliminary result, we concluded that Drep2 exists as a highly oligomeric form mediated by CIDE domain oligomerization in solution, unlike Drep3, which usually exists as a monomer in solution. This result also indicates that the CIDE domain, which is a protein interaction module involved in hetero-dimerization, can be self-oligomerized.

3.2. Drep2 interacts with Drep3 via the CIDE domain

Purified recombinant Drep2 and Drep3 proteins and their CIDE domains were used for an analysis of their interactions. To analyze the interaction of Drep2 with Drep3, purified Drep2 CIDE was

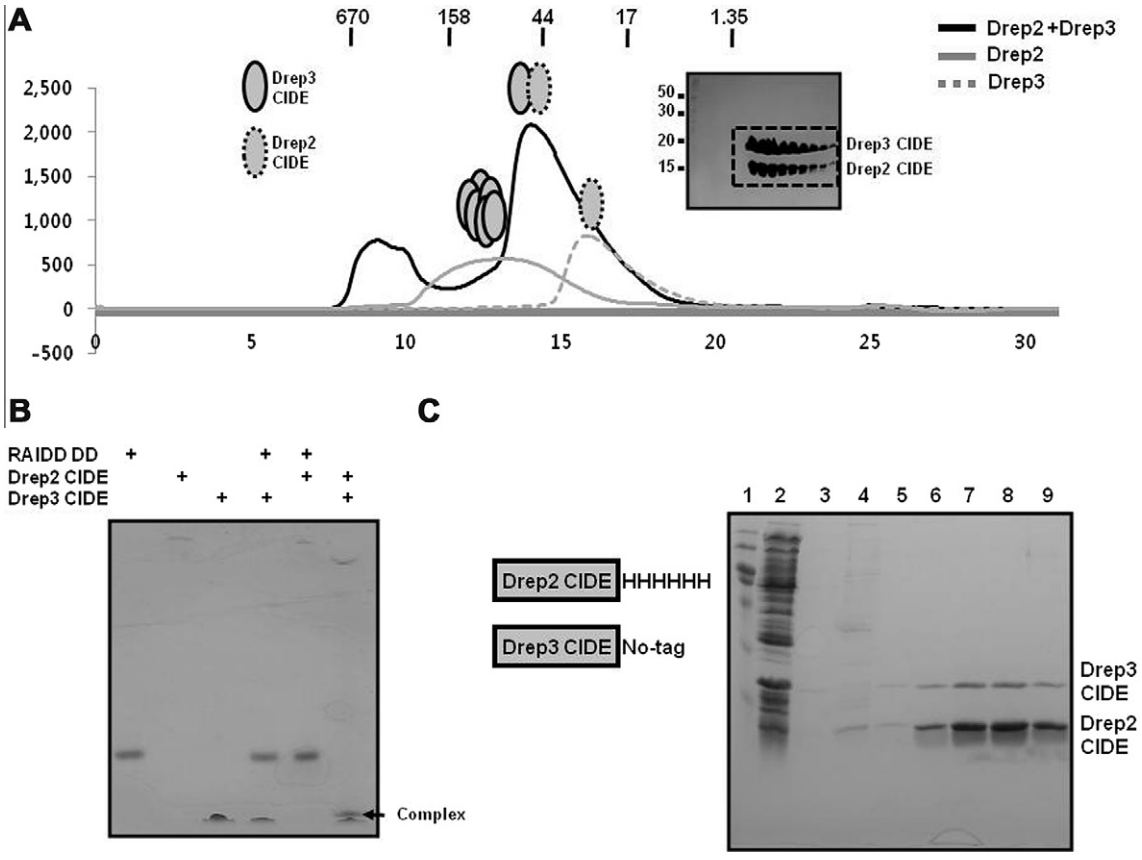

Fig. 2. Drep2 interacts with Drep3 via the CIDE domain. (A) Gel filtration chromatogram and fractions of a mixture of Drep2 CIDE and Drep3 CIDE. Black line: Drep2 CIDE and Drep3 CIDE mixture, Gray dot: Drep3 CIDE, Gray line: Drep2 CIDE. SDS–PAGE of the peak gel-filtration chromatography fractions from the mixture of Drep2 CIDE and Drep3 CIDE is shown at right side. The complex fraction is indicated by the dotted-box. (B) Native-PAGE analysis of the interaction between Drep2 CIDE and Drep3 CIDE: Lane 1, RAIDD DD only (negative control); lane 2, Drep2 CIDE only; lane 3, Drep3 CIDE only; lane 4, Drep3 CIDE mixed with RAIDD DD; lane 5, Drep2 CIDE mixed with RAIDD DD; lane 6, Drep2 CIDE mixed with Drep3 CIDE. The complex band is indicated by arrow. (C) Co-expression and His-tag pull-down of Drep3 CIDE-noHis by His-tagged Drep2 CIDE. Lane 1, markers; lane 2, supernatant of the cell lysate; lane 3, flow through after incubation with Ni-NTA beads; lane 4, washing buffer containing 20 mM imidazole; lanes 5–9, 250 mM imidazole eluted fractions.

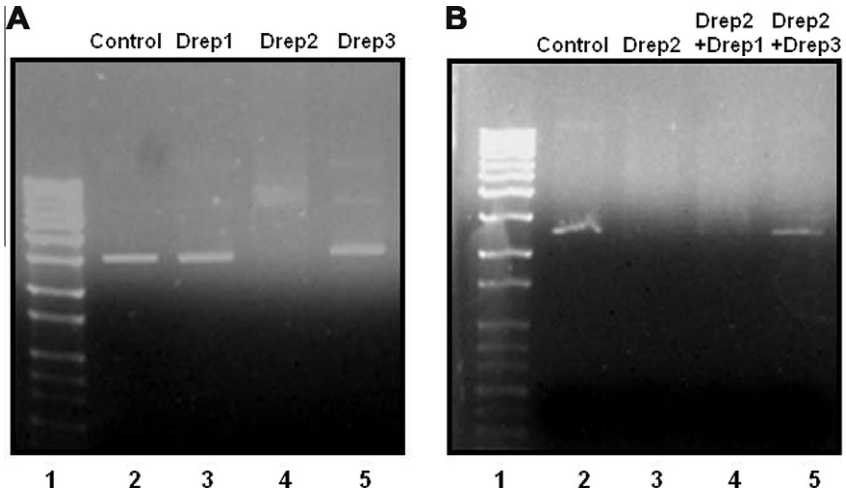

Fig. 3. Drep2 is a nuclease that is inhibited by Drep3. (A) Nuclease activity assay shows degradation of linearlized plasmid DNA. Lane 1, DNA markers; lane 2, DNA with water (negative control); lane 3, DNA with purified Drep1; lane 4, DNA with purified Drep2; lane 5, DNA with purified Drep3. (B) Nuclease inhibitory assay shows blocking degradation of linearlized plasmid DNA. Lane 1, DNA markers; lane 2, DNA with water (negative control); lane 3, DNA with Drep2; lane 4, DNA with the Drep2 and Drep1protein mixture; lane 4, DNA with a mixture of Drep2 and Drep3.

mixed with Drep3 CIDE, and each sample was incubated for 1 h at room temperature and then applied to a S-200 gel-filtration column. The Drep2 CIDE:Drep3 CIDE complex was obtained, as shown by gel filtration chromatography and SDS–PAGE (Fig. 2A). An obviously shifted peak and co-migration on SDS–PAGE indicated that Drep2 CIDE interacts tightly with Drep3 CIDE (Fig. 2A). These interactions were also confirmed by native-PAGE (Fig. 2B). Although the mixture of Drep2 CIDE and the RAIDD death domain (RAIDD DD, used as a negative control) or Drep3 CIDE and RAIDD DD did not produce a complex band, the mixture of Drep2 CIDE and Drep3 CIDE created a new complex band. We conducted a His-tag pull-down assay using Drep3 CIDE without the His-tag to confirm the interaction between Drep2 and Drep3. Co-expressed bacterial cell pellets were resuspended, homogenized, and fractionated into a soluble supernatant fraction and an insoluble fraction. The soluble fraction, which contained both His-tagged Drep2 CIDE and Drep3 CIDE without the His-tag, was incubated with Ni-NTA beads. After extensive washing, the beads were eluted using imidazole, and the eluted fractions were examined by SDS–PAGE. As indicated in Fig. 2C, Drep3 CIDE was pull-downed and co-migrated with Drep2 CIDE on SDS–PAGE, indicating that Drep2 CIDE interacts with Drep3 CIDE.

3.3. Drep2 is novel nuclease of which activity is inhibited by Drep3

Because the biochemical behavior of Drep2 was similar with that of DFF40 and Drep4, which possess nuclease activity, we analyzed for possible Drep2 nuclease activity using an agarose gel with linearized plasmid DNA. Drep2 activity can be assayed by its ability to digest plasmid DNA. While linearized plasmid DNA incubated with water or Drep1 as a negative control was not capable of degrading DNA, DNA incubated with Drep2 was clearly digested (Fig. 3A). Drep3 was not able to digest DNA (Fig. 3A).

After analyzing the interaction between Drep2 CIDE and Drep3 CIDE and Drep2 nuclease activity, we wondered if the interaction between Drep2 and Drep3 via the CIDE domain modified Drep2 activity. Thus, we conducted the same nuclease activity assay performed above using a Drep2 and Drep3 mixture. As shown in the Fig. 3B, although the Drep2 and Drep1 mixture was unable to block Drep2 nuclease activity, Drep3 completely blocked DNA digestion, indicating that Drep2 has nuclease activity and that the nuclease activity of Drep2 was inhibited by Drep3 through a direct interaction.

3.4. Drep3 is cleaved by caspase-1 (Dcp1) and may be involved in apoptotic DNA fragmentation

Interestingly, Drep3 contained the fly caspase-1 (DCP-1) cleavage site (Fig. 4A). This observation suggests that Drep3 can be cleaved by DCP-1 during apoptosis signaling, dissociated from Drep2, and cause DNA fragmentation. This activity controlling system is similar with that of the DFF40/DFF45 system found in mammals.

Taken together, we suggest that two apoptotic DNA fragmentation systems occur in the fly: theDrep1:Drep4 and Drep2:Drep3 systems. Drep4 and Drep2 are nucleases and their activity is inhibited by Drep1 and Drep3, respectively. Although we were unable to analyze the direct involvement of DCP-1 in this Drep2/Drep3 complex system due to the unavailability of active DCP-1 protein, which is insoluble in the solution, the Drep2:Drep3 system may

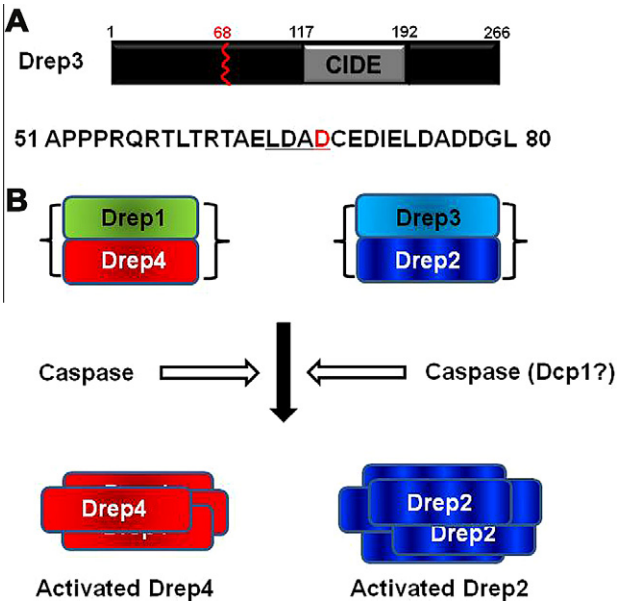

Fig. 4. Drep3 is cleaved by caspase-1 (Dcp1) and may be involved in apoptotic DNA fragmentation. (A) Predicted caspase-1 cleavage site on Drep3. Possible cleavage sites are indicated by the red line. The prediction was made by CASVM server 1.0 (http://www.casbase.org/casvm/server/index.html). (B) Model for the dual apoptotic DNA fragmentation system in the fly.

be activated by Drep3 cleavage by DCP-1. The possible chaperone activity of Drep3 and the involvement of DCP-1 in this system should be examined in the future.

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